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| STANDARD OPERATING PROCEDURE |
| |  |  | | --- | --- | | **Title: Cell Lysis, Tryptic Digestion, and Phosphopeptide Enrichment by Automated Immobilized Metal Affinity Chromatography (IMAC)** | | |  |  | | **Version #: 2** | **Author: Broad Inst Proteomics Platform** | | **Date: June 30, 2016** | **BRD-001** | |

# Purpose

The purpose of this document is to describe cell lysis, tryptic digestion, and phosphopeptide enrichment by IMAC of samples for analysis by mass spectrometry.

# Scope

This procedure may be used to make a cell lysate, prepare a tryptic digest, and enrich for phosphopeptides.

# Responsibilities

It is the responsibility of person(s) performing this procedure to be familiar with laboratory safety procedures. The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation.

# Equipment

1. Microcentrifuge
2. Benchtop vortex mixer
3. Incubator
4. Plate Reader
5. Agilent Bravo Automated Liquid-Handling Robot with AssayMAP head

# Materials

1. tC18 SepPak cartridges(Waters, 500 mg WAT036790)
2. Agilent Bravo AssayMAP Fe(III)-NTA cartridges (Agilent #G5496-60085)
3. Agilent Bravo AssayMAP Reversed Phase (RP-S) cartridges (Agilent # G5496-60033)

# Reagents

**Cell Lysis Reagents:**

1. Urea
2. NaCl
3. Tris
4. EDTA
5. Aprotinin (Sigma, A6103)
6. Leupeptin (Roche, #11017101001)
7. PMSF (Sigma, 78830)
8. NaF
9. Phosphatase Inhibitor Cocktail 2 (Sigma, P5726)
10. Phosphatase Inhibitor Cocktail 3 (Sigma, P0044)
11. HPLC water

**BCA Assay**

1. BCA assay (Pierce, 23227)

**Protein Reduction, Alkylation and Digestion**

1. Dithiothreitol (Thermo Scientific, 20291)
2. Iodoacetamide (Sigma, A3221)
3. Tris HCl, pH 8.0
4. Trypsin (Promega, V511X)
5. LysC (Wako, 125-05061)
6. Formic acid (Fluka, 56302)
7. HPLC water

**Desalt**

1. Acetonitrile
2. Formic acid
3. Trifluoroacetic acid (Fluka, TX1276-6)
4. HPLC water

**Automated IMAC Enrichment Solutions**

1. Acetonitrile
2. Methanol
3. Acetic acid
4. Trifluoroacetic acid (Fluka, TX1276-6)
5. K2HPO4
6. HPLC water

**Automated IMAC Enrichment – Desalt Solutions:**

1. Acetonitrile
2. Trifluoroacetic acid (Fluka, TX1276-6)
3. HPLC water

# Solutions

**Cell Lysis Solutions**

1. Lysis Buffer – For lysis of 10^6 suspension cells use at least 1 mL lysis buffer (to obtain a protein concentration < 5 mg/mL)

8 M Urea

75 mM NaCl

50 mM Tris pH 8.0

1 mM EDTA pH 8.0

*Add immediately before use the following additives:*

2 µg/mL Aprotinin (1:500 of 1 mg/mL in water)

10 µg/mL Leupeptin (1:200 of 2mg/mL in water )

10 mM NaF (1:100 of 1 M stock in water )

PIC3 (1:100 Phosphatase inhibitor cocktail 1)

PIC2 (1:100 Phosphatase inhibitor cocktail 2)

1 mM PMSF (1:100 of 100 mM stock in ethanol)

Ex. For 5mL lysis buffer, add:

2.4g urea

750 uL 1M NaCl

500 uL 1M Tris pH 8.0

20 uL 500mM EDTA pH 8.0

~4.5m L water

*Add immediately before use the following additives:*

20 uL 2 µg/mL Aprotinin (1:500 of 1 mg/mL in water)

50 uL 10 µg/mL Leupeptin (1:200 of 2mg/mL in water)

100 uL 10 mM NaF (1:100 of 1 M stock in water)

100 uL PIC3 (1:100 Phosphatase inhibitor cocktail 1)

100 uL PIC2 (1:100 Phosphatase inhibitor cocktail 2)

100 uL 1 mM PMSF (1:100 of 100 mM stock in ethanol)

**Digest Solutions**

1. 5 mM DTT
2. 10 mM IAA
3. 10% Formic Acid

**Desalt Solutions**

1. 50 mM Tris HCl, pH 8.0
2. 50 % acetonitrile/0.1% formic acid
3. 1% formic acid
4. 0.1% trifluoroacetic acid

**Automated IMAC Enrichment – pSTY Enrichment Solutions**

1. 1:1:1 ACN:MeOH:0.01%AceticAcid
2. 80% ACN / 0.1% TFA
3. 500 mM K2HPO4, pH 7

**Automated IMAC Enrichment – Desalt Solutions:**

1. 80% ACN/0.1% TFA
2. 50% ACN/ 0.1% TFA
3. 0.1% TFA

# Procedure

**Cell Lysis:**

1. Lyse cells (1E6) at 4°C w/ chilled 1mL lysis buffer for target concentration of less than 5 mg/mL protein for 30 min on wet ice. Vortex occasionally.
2. Centrifuge at 20,000 x g to remove cell debris at 4°C for 15 min. Transfer supernatant to another tube.
3. Measure protein concentration using BCA.

**In solution-digest:**

1. Reduce denatured proteins with 5 mM DTT for 30 min at 37°C .
2. Alkylate proteins with 10 mM IAA for 45 min at room temperature in the dark.
3. Dilute sample 1:4 with 200 mM Tris pH 8.0 to decrease urea concentration below 2 M.
4. Add endoproteinase Lys-C (Wako) to an enzyme to substrate ratio of 1:50 and incubate at 800 RPM at 37°C for 2h.
5. Add trypsin (Promega) to an enzyme to substrate ratio of 1:50 and incubate at 800 PRM at 37°C overnight (~ 16h).
6. Add formic acid to 10% to acidify the digest to pH 1.5-2 to quench enzymatic activity.

**Desalt via tC18 SepPak (Waters, 500 mg WAT036790)**

1. Condition cartridge with 5 mL 100% acetonitrile followed by 5 mL 50% acetonitrile / 0.1% FA.
2. Equilibrate with 4 x 5 mL of 0.1% TFA.
3. Load sample.
4. Wash/desalt with 3 x 5 mL of 0.1% TFA.
5. Wash/desalt with 1 x 5mL of 1% FA (to remove TFA).
6. Elute with 2 x 3mL of 50 % acetonitrile / 0.1 % FA.
7. Aliquot to 5 mg/tube in 2 mL tubes (Sarstedt).
8. Freeze eluate with liquid N2 (or at -80°C) and lyophilize (or speed-vac) to dryness and store in -80°C freezer.

**Sample Plate Preparation**

1. Thaw and resuspend a pellet of digested desalted cell lysate (5 mg/tube) in 1 mL 50 %ACN/ 0.1% TFA. Vortex thoroughly and centrifuge briefly (20 s at 2000 x g).
2. Add 1.5 mL 100% ACN/ 0.1% TFA. Transfer to 15 mL tube (Falcon). Repeat vortexing and centrifugation. Samples are now at 80% ACN/ 0.1% TFA.

**Automated IMAC Enrichment using Fe(III)-NTA AssayMAP cartridges and Desalt using AssayMAP Reversed Phase (RP-S) cartridges**

**Automated IMAC Enrichment Steps (Agilent Workbench Protocol: Phosphopeptide\_enrichment\_v2.0)**

1. Prime Fe(III)-NTA cartridges with 100 uL of 1:1:1 (*100 uL/min*).
2. Equilibrate cartridges with 80% ACN/0.1% TFA.
3. Load sample in dispense mode (*5 uL/min).*
4. Wash cup with 25 uL 80% ACN/0.1% TFA .
5. Wash sample with 50 uL 80% ACN /0.1% TFA (*10 uL/min*).
6. Stringent syringe wash with 50 uL 500 mM K2HPO4.
7. Elute peptides from cartridges with 50 uL of 500 mM K2HPO4 (*5 uL/min*).
8. *(Manual)* Freeze flowthrough at -80°C. Remove the IMAC cartridges, place in a labeled box and store dry at room temp.

**Automated Desalt Protocol**

1. Prime RP-S cartridges with 50 uL 80% ACN/0.1% TFA (*10 uL/min*).
2. Equilibrate RP-S cartridges with 50 uL 0.1% TFA (*10 uL/min*).
3. Load sample in dispense mode (*2 uL/min*).
4. Wash cup with 25 uL 0.1% TFA.
5. Wash sample with 50 uL 0.1% TFA (*10 uL/min*).
6. Wash syringe with 50 uL 50% ACN/0.1% TFA.
7. Elute peptides with 50 uL 50% ACN/0.1% TFA (*2 uL/min*).
8. *(Manual)* After protocol completes, cover flowthrough plate with a foil sealmat. Freeze at -80°C.
9. *(Manual)* Transfer each sample into autosampler vials. Freeze at -80°C.
10. *(Manual)* Speedvac autosampler vials to dryness.

# Referenced Documents

For complete details of protocols for cell lysis, digestion and automated IMAC enrichment:

[Mol Cell Proteomics.](http://www.ncbi.nlm.nih.gov/pubmed/26912667) 2016 May;15(5):1622-41. doi: 10.1074/mcp.M116.058354. Epub 2016 Feb 24.

**Reduced-representation Phosphosignatures Measured by Quantitative Targeted MS Capture Cellular States and Enable Large-scale Comparison of Drug-induced Phenotypes.**

PMID:26912667

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